United States Environmental Protection Agency

LABORATORY ANIMAL PROJECT REVIEW

Please note:

1. All information in this LAPR is considered privileged and confidential by the IACUC and regulatory authorities.
2. Approved LAPRs are subject to release to the public under the Freedom of Information Act (FOIA). Do not include proprietary or classified information in the LAPR.
3. An approved LAPR is valid for three years.

LAPR Information

LAPR Title:	**EFFECT OF PRENATAL EXPOSURE TO OZONE ON MATERNAL RESPONSE AND RISK OF PRE-ECLAMPSIA IN RATS.**
LAPR Number:	**18-08-003**
Principal Investigator	**Exemption 6**
Author of this Document:	**Exemption 6 /RTP/USEPA/US**
Date Originated:	**08/24/2015**
LAPR Expiration Date:	**08/31/2018**
Agenda Date:	**09/09/2015**
Date Approved:	**09/17/2015**
Date Closed:	

APPROVALS

APPROVER	NAME	APPROVAL DATE	COMMENTS	
	Exemption 6 /RTP/USEPA/US by Exemption 6 /RTP/USEPA/US	09/17/2015	DMR	
	Exemption 6 Exemption 6 Exemption 6 RTP/USEPA/US by Exemption 6 /RTP/USEPA/US	09/17/2015	DMR	

Administrative Information

1. Project Title (no abbreviations, include species):
EFFECT OF PRENATAL EXPOSURE TO OZONE ON MATERNAL RESPONSE AND RISK OF PRE-ECLAMPSIA IN RATS.

Is this a continuing study with a previously approved LAPR?
No

2. Programatic Information
a. What Program does this LAPR support? Please provide the Research Program, Project, Task Number and Title.
ACE, PEP1 (Tasks 1 and 2); PEP2 (Task2)
b. What is the Quality Assurance Project Plan (QAPP) covering this project?
IRP-NHEERL/EPHD/CIB/2015-01-01r0

3. EPA Principal Investigator/Responsible Employee:

Principal Investigator	*Phone Number*	*Division*	*Mail Drop*
Exemption 6	Exemption 6 *Lotus Notes Address* Exemption 6/RTP/USEPA/US	EPHD *Branch* CIB	MD

4. Alternate Contact:

Alternate Contact	*Phone Number*	*Division*	*Mail Drop*
Exemption 6	Exemption 6 *Lotus Notes Address* TP/USEPA/US	EPHD *Branch* CIB	MD

SECTION A - Description of Project

1. Explain the study objective(s) in non-technical language such that it is understandable by non-scientific persons. Explain how the benefits from the knowledge gained from this research outweigh the costs to the animals used in this research. If this is a continuing study from a previous LAPR, briefly justify the

continuation. Please spell out all acronyms and abbreviations with their initial use.

The first 1,000 days of life in humans has been identified as a period where adult-disease risk is developed. This time period expands through the entirety of gestation and through lactation in children. Stressors that occur during this critical window, which can include poor nutrition, emotional stress, and exposures to environmental agents including air pollutants, can increase the risk of numerous defects and diseases in the offspring. Further, exposures to stressors during gestation may increase the risk of developmental/gestational disorders including poor fetal growth, gestational diabetes, and pre-eclampsia.

Pre-eclampsia is a spontaneous development of maternal high blood pressure during gestation that is life-threatening to both the mother and the child. While the prevalence of pre-eclampsia is relatively common, affecting up to 3 million mothers in the United States annually, it is referred to as spontaneous because very little is known about its development and risk factors associated with it. Research in humans has suggested that higher exposures to air pollutants increase the risk of pre-eclampsia and poor fetal growth. While the literature provides intriguing links to air pollution and pre-eclampsia, such links are purely observational and fail to confidently conclude that exposure to pollution during pregnancy can result in life-threatening complications such as pre-eclampsia.

In this project we will monitor the development of the known hallmarks of pre-eclampsia in rats exposed to ozone throughout pregnancy. This will include the monitoring and measurement of tail blood pressure, protein excretion in the urine, and placental blood flow as measured by ultrasound. We will also measure blood glucose concentrations as an indicator of gestational diabetes as its development is a known risk-factor of pre-eclampsia. Pregnant rats will be euthanized near the end of gestation, day 21, so that the fetal weight can be measured and tissue can be collected, including the placenta which could not be obtained if pregnant rats were permitted to give birth. These studies may provide the first causative evidence that gestational ozone exposure elevates the risk of pre-eclampsia and rationale for the development of risk assessment and mitigation strategies to reduce the effects of pollution in this potentially sensitive population.

2. Scientific rationale for proposed animal use.

a. Why is the use of animals necessary?

Because pre-eclampsia can lead to life-threatening complications it would be unethical to expose pregnant humans to ozone even in an acute manner. As the current relationship between air pollution and pre-eclampsia is purely observational and little is known about the mechanisms and risk stratifications, exposing a pregnant human may result in a life-threatening situation. This risk makes it necessary to research the potential causative relationship of air pollution and pre-eclampsia in an animal model. Further, the use of an animal model allows for the undertaking of mechanistic studies including the change to the placenta during gestation which may provide important information on the imprinting of disease risk in the offspring. This complex systemic responses that culminate in pre-eclampsia cannot be measured by cell culture techniques and thus, again requires the use of an animal model.

b. Justify the species requested:

Rats have been the preferred animal model for the study of cardiovascular injury and risks from air pollution. This is supported by the NIH guidelines that support the use of rats for cardiovascular and metabolic studies. Due to the longstanding use of rats for toxicological, cardiovascular, and metabolic studies the necessary databases, reagents, and species-specific assays have been developed, verified to be accurate, and are commercially available.

3. How was it determined that this study is not unnecessary duplication?

Pubmed and literature searches in Google Scholar performed in July and August 2015 failed to identify any published studies that investigated pre-eclampsia in pregnant rats exposed to ozone by searching the terms “ozone,” “pre-eclampsia,” and “rat” or “mouse.” An additional search that included “air pollution” and “placenta” identified one study that investigated the morphological changes of placental blood supply following gestational and pre-gestational exposure to particulate matter in mice. Five other rodent studies existed that demonstrated reduced placental and fetal weights, changes to placental morphology, and increased inflammation with inhaled air pollutants. No studies investigated pre-eclampsia under the broader term of “air pollution.” Lastly, components of pre-eclampsia including elevated urine proteins levels (“proteinuria”), “blood pressure,” and “fetal growth” were also searched alongside the terms “pregnancy” and “ozone.” Similarly to pre-eclampsia, no studies in rats were identified.

SECTION B - In Vivo Procedures

1. Briefly describe the experimental design. Include descriptions of the age, weight and sex of the animals. Supplementary information may be attached at the end of the LAPR, but please include critical information within the body of the LAPR.

This project will examine components of pre-eclampsia in pregnant rats exposed to ozone throughout pregnancy. For tabulated experimental design, please refer to the attachment.

Timed-pregnant, adult Long-Evans rats will be purchased from Charles Rivers and will arrive at our facility at gestational day 3. Pregnant rats will be maintained on a fat-modified, purified diet (D12450H; Research Diets, Inc., New Brunswick, NJ). This diet will be modified to increase the fat content from 10 kcal% to 16 kcal% to meet the recommended macronutrient levels for growth and gestation developed by the American Institute of Nutrition Rodent Diets committee. No other modifications will be made. This diet is free of phytoestrogens which are known to impact the reproductive, cardiovascular, and metabolic systems. As these systems play a role in the development of pre-eclampsia, removal of phytoestrogens from the diet reduces this confounding factor.

Two experimental objectives will be performed in this study.

a. Objective 1: optimization of the assays through the use of a positive pre-eclampsia induction treatment. It is anticipated that up to 20 imaging sessions will be necessary to optimize the ultrasound technique sufficient to acquire consistent placental blood flow measurements. To achieve this, two groups of 10 rats will be used to first become familiarized with the assays and secondly to be used to obtain experimental data. For the purpose of the positive control assessment, rats will be randomized into two groups (n=10/ group). Starting from gestational day 14, rats will either receive 100 mg/L of the nitric oxide inhibitor N-Nitro-L-arginine methyl ester (L-NAME) delivered with the drinking water or tap water in the case of the control rats. This treatment protocol has been identified to produce a hypertensive response that results in the development of the hallmarks of pre-eclampsia. In total, 40 pregnant rats will be used during this phase. Following Objective 1, we will begin Objective 2.

b. Objective 2: the assessment of the effects and critical windows of ozone exposure on markers of pre-eclampsia during gestation. A group pregnant rats will serve as a cage control group that will not receive exposure to ozone or air in the whole body chamber system (Group 1; n=10). The inclusion of a cage control group allows for the investigation of the role of the exposure model on the outcomes in this study. This group will run separately, but interspersed with the ozone-exposed rats. Four exposure groups will be used during this objective. Groups 2 and 3 will be exposed to air or 0.8 ppm ozone (n=10/ group) for 4 hours/day for 16 consecutive days from gestational day 6 to 21. Groups 4 and 5 (n=10/ group) will be exposed in a cross-over design where pregnant rats will be exposed to air or 0.8 ppm ozone for 4 hours for 8 consecutive days during gestational days 6 to 13. The groups will then be switched and receive the air or 0.8 ppm ozone exposure for 4 hours for the following 8 days during gestational day 14 to 21. Due to the length of the study and numbers required, it will be ran in 4 blocks with the exposure groups evenly dispersed among them. The total number of rats to be used in this phase is 50 (5 groups x 10 rats/group).

For the above experimental objectives, markers of pre-eclampsia will be monitored including tail blood pressure and concentration of urine metabolites. Baseline measurements will be obtained prior to ozone exposure (gestational day 6), during a midpoint of exposure (gestational day 13), and on gestational day 20 (the day prior to euthanasia). This data will be correlated with uterine and placenta blood flow that will be measured by echocardiogram/ ultrasound.

Please refer to the attached document for additional dietary information, a table of groups, and study design.

2. Justify the number of animals. Include explanation (e.g., biological, statistical, regulatory rationale) for the number of animals needed for each treatment group, and the overall number requested for the duration of the LAPR.

A total of 90 pregnant rats Long-Evans rats are proposed to be included within this study. During the phase of optimization, a total of 40 animals are necessary to allow for the ultrasound scientist to obtain the confidence

required to complete the assessments on the effects of ozone. Ten rats will be used for exploration and optimization of the protocol to obtain uterine and placental blood flow; an additional naïve cohort of 10 rats will be needed to establish mean and SEMs for all endpoints. Lastly, the final 20 rats will be used in a positive control experiment (2 groups; n=10/ group) that will permit the identification of poor uterine blood flow following a known chemical that induces pre-eclampsia in rats (L-NAME). Proper optimization reduces technical error and permits for reduced numbers of rats in the following studies.

To assess the effects of ozone on the risk of pre-eclampsia we will use 5 total treatment groups, including a cage-control, air-control, prolonged ozone exposure, and a cross-over design that permits for internal control to determine the effects of ozone exposure in early and late pregnancy. To achieve the necessary statistical power involving standard analysis of variance techniques, an n=10 rats per group are requested. With 5 total treatment groups, this results in an n=50 animals. Because pregnancy cannot be 100% assured by Charles Rivers (90% confidence is reported at gestational day 3), our expected number of rats required reflect this possibility. Our numbers additionally protects the statistical integrity if ozone exposure results in spontaneous abortion in a pregnant rat. Therefore, we propose the use of 50 pregnant rats during Objective 2. Together with Objective 1, this brings the proposed total number of timed-pregnant rats to 90.

The repeated measures and monitoring assessments in the rats during this study reduces the need to include multiple sets of animals. Further, the robust amount of tissues that will be obtained and preserved allow for a variety of ex vivo analyses. As the pregnant rats will be euthanized at gestational day 21 and thus will not be permitted to deliver, we will be able to obtain a large library of tissues from fetuses that reflect the pre-natal window of exposure. Such samples can be compared with later research that involves early life exposures to ozone and adult disease risk and will minimize the need to repeat such experiments in the future.

To assure that we reach the appropriate numbers to achieve statistical significance, this experiment will require 90 pregnant rats (n=10/ group). Studies investigating pre-eclampsia in a rat model range in the number of replicates typically used between 6 – 8 rats. Because of the possibility of pregnancy loss induced by ozone exposure and the potential of false positive pregnancy, we propose the use of 10 pregnant rats for each group within this study. Thus, this brings the total number to 90 rats.

3. State how many animals over the study period are expected to be used under the following three categories of pain/distress (USDA nomenclature as defined in the instructions): Please enter numbers only.

Categories	Adults	Offspring
C) Minimal, transient, or no pain/distress:	90	
D) Potential pain/distress relieved by appropriate measures:		
E) Unrelieved pain/distress:		

4. Does this LAPR include any of the following:

- ☐ Restraint (>15 Minutes)
- ☐ Food and/or water restriction (>6 Hours)
- ☐ Survival surgery
- ☐ Non-survival surgery

5. Category C procedures. Describe each procedure separately, include details on the following:

a. Treatments (e.g., dosages, duration of exposure, route, volume, frequency):

L-NAME:

L-NAME will be used to induce pre-eclampsia in pregnant rats. L-NAME at a dose of 100 mg/L will be delivered via drinking water to an n=10 rats during Objective 1. Control rats will be provided with drinking water without L-NAME. Water will be provided ad libitum in glass water bottles and will be replenished every 3 days, or earlier as needed. Rats will be treated from gestational day 14 through euthanasia at day 21. Based off of published research L-NAME administration in the drinking water in the latter half of pregnancy will reliably elevate proteinuria and blood pressure within 2 – 4 days of initiation (Murphy et al.; Am J Physiol Regul Integr Comp Physiol. 2012). While L-NAME adequately induces pre-eclampsia in the rat it does not result in severe, life threatening outcomes.

General whole body inhalation exposure conditions:

During ozone inhalation exposures of 4 hours/ day (whole-body), rats are housed in stainless steel wire mesh cages. Food and water are withheld while rats are being exposed. Rats will be weighed after each ozone exposure, and examined for any visible clinical signs of discomfort or poor health. All findings are recorded. Ozone exposures will be done at 0.8 ppm concentration for a period of 4 hours per day for either 8 or 16 days consecutively using the whole body exposure system. The dose was selected based off of previous work by Gunnison and Hatch (Am J Physiol. 1999).

Control rats will be exposed to filtered air under the same conditions alongside ozone exposed rats.

b. Survival Blood Collections (method, volume, frequency):

n/a

c. Testing methods (including non-stressful dietary restrictions/modifications, mild non-damaging electric shock):

1) Urine: The collection of urine from the pregnant rats throughout the study permits for the monitoring of glucose levels in the urine (glycosuria) which is an indicator of diabetes. Further, proteinuria (excessive protein in the urine) can also be monitored through the collection of urine and is an indicator of pre-eclampsia. The protocol, as described below, is non-invasive and causes no stress to the animal. The timing of the urine collection will be uniform throughout the study. For example, if urine is collected between 8:00 – 10:00 AM on the baseline day, it will be collected at this same time for the mid and endpoint analyses. Urine will be collected at gestational days 6, 14, and 20 for both Objective 1 and 2 and thus will adequately span baseline, midpoint, and endpoint measurements. In the case of L-NAME (Objective 1), both gestational day 6 and 14 will be considered as baseline/ non-treatment points.

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Urine collection protocol: A pregnant rat will be placed into a clean, Plexiglas cage without bedding. The rat is allowed to urinate freely without invasive intervention by the researcher. Following each urination, the urine will be collected using a pipette into a microcentrifuge tube, which will be placed on ice between collections. The researcher will attempt to collect at least 500 µl prior to returning the animal to their home cage and obtain a glucose reading using a urinary glucose test strip with a small sample of urine. In between rats, the cage will be rinsed and cleaned using 70% ethanol.

2) Tail blood pressure: Rats will undergo measurement of blood pressure using a non-invasive tail cuff as described in OP-NHEERL-H/EDT/IEG/01-44-00 and below. As blood pressure is sensitive to circadian rhythms and stress, the daily timing of the blood pressure assessment will be uniform throughout the study. The measurement of tail blood pressure requires transient restraint in a tube. However, rats are permitted multiple acclimation periods to the restraint tubes (2-3 times for approximately 15 minutes each) prior to beginning the experiment.

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Tail blood pressure protocol: The warming chambers should be turned on several hours prior to use to stabilize the temperature at about 32º C. The larger warming chamber is used for pre-warming the rats and the smaller chamber used for the actual blood pressure measurement. Only one rat at a time is placed in the smaller chamber to avoid noise distraction during the measurements. Each rat's blood pressure is measured at least 5 times to get an accurate reading. Additional measurements are taken if the rat moves during the test.

The rat may remain in the warming chambers for up to 15-20 minutes.

A rat is placed in the restraint tube and the tail cuff is placed over the tail as close to the base of the tail as possible. The rat is placed in the large warming chamber for about 6-9 minutes to increase the body temperature enough to cause the rat to begin regulating body temperature by dilating blood vessels in the tail. A photoelectric sensor in the tail cuff senses pulses as the blood flows through the tail.

The rat is then moved to the smaller chamber and the tail cuff is plugged into the Blood Pressure Analyzer via the built-in scanner. The animal's identification is entered into the software along with the number of measurements to take. The start button is pressed and the tail cuff compresses to 300 mmHg to stop blood flow in the tail. The pressure is then released slowly and the pressure when pulses are detected is recorded. Heart rate, Systolic and Mean pressure are recorded and Diastolic pressure calculated. After testing the rat is returned to its home cage.

3) Ultrasound: At 3 separate time points during pregnancy, including the morning of gestational day 21, rats will briefly undergo light isoflurane anesthesia to obtain data on uterine/placenta blood flow, heart rate (maternal and fetal), and maternal cardiac function by the use of non-invasive ultrasound echocardiography. The rats are kept on a heated platform for thermoregulatory support of core body temperature during anesthesia and their heart rate and breathing will be constantly monitored. While ultrasonography does not result in any pain, it is necessary for the rat to be placed in dorsal recumbency and to remain in the same position during imaging. Therefore, we will use isoflurane to induce a light plane of anesthesia. We are limiting our measurement to 3 maximum for a limited amount of time (< 30 minutes) during each assessment. Thus, there will be minimal impact of isoflurane, anesthesia, or stress on the developing fetus or overall maternal health of the dam.

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Ultrasound protocol:

Rat anesthesia will be induced by placing the rat in an uncharged induction chamber that will be filled with 3% isoflurane (O2 flow at 0.8-1.0 L/min). The induction chamber, isoflurane unit, and ultrasound platform will be kept underneath a fume hood to reduce gas waste and unnecessary exposure of isoflurane to staff and waiting rats. After induction, rats will be transferred to a heated platform (for thermoregulatory support of core body temperature) and anesthesia will be maintained using 1%-3% isoflurane in oxygen delivered at 0.8-1.0 L/min delivered via nose cone. Artificial tear ointment will be added at this time to protect the rat's eyes during anesthesia. Heart rate and respiration rate will be closely monitored throughout the protocol. Since fur/hair interferes with ultrasound, hair will be removed from the thorax and abdomen with electric clippers followed by application of a depilatory agent (e.g. Nair). Due to the caustic nature of depilatory agents, care will be taken to remove and clean the abdomen of the depilatory agent following hair removal. A pre-warmed ultrasound gel will be applied to the abdomen during the ultrasound procedure. Ultrasound will be used to image fetuses and arteries within the abdomen of the pregnant dam, as well as the heart of the pregnant dam. These data will be used to determine heart function parameters in both the dam and the fetuses, and if possible determine blood flow velocities within the arteries of interest (uterine, arcuate, radial, and spiral). On gestational day 21, immediately following the measurements,

rats will be removed from isoflurane and a terminal dosage of pentobarbital will be injected intraperitoneally (maximum 150 mg pentobarbital/kg).

d. Animal restraint and confinement beyond routine housing and handling. Include a description of the type of restraint device, acclimation to device, duration of restraint:

For the purposes of blood pressure assessment, pregnant rats will be placed into a size appropriate Plexiglas tube restrainer. The length will be individually adjusted to accommodate the length of the animal. Rats will be introduced to the tube on gestational day 5, 24 hours prior to the first blood pressure assessment. To reduce the stress of the animal and help maintain body temperature, rats will be kept at 31-32ºC immediately prior to and during the experiment. Rats are restrained following a pre-warming period in an appropriate sized tubes and placed back into a warming chamber for 6-9 minutes. Blood pressure is then monitored for 5 cycles of compression and relaxation. The total duration of restraint is approximately 15 minutes. Blood pressure will be obtained on gestational days 6, 13, and 20.

e. Breeding for experimental purposes (e.g. length of pairing, number of generations):

none

f. Describe how animals will be identified and monitored. Include description of identification procedures. (For example, if transponders are used, how are the animals prepared?) Include frequency of observations and by whom:

During exposure **Exemption 6Exemption 6Exemption 6** will monitor animals, at least once per hour for entire exposure duration. During non-exposure periods, rats will be monitored once daily for visible signs of discomfort. Food intake and body weight will be obtained on a daily basis by **Exemption 6**, inclusive of weekends and holidays. No weight loss or significant distress is expected with any of the experimental conditions. Animals will be identified through tail marks of their unique identification number. The marking will be rewritten throughout the study as needed.

6. Non-surgical Category D or E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9).

a. Treatments (e.g. dosages, duration of exposure, route, volume, frequency):

None

b. Blood Collection (Provide a description of the procedure including method, volume, and frequency if appropriate. Indicate if the procedure is survival or terminal. Include preparatory methods, descriptions of incisions, etc.):

None

c. Testing methods:

n/a

d. Restrictions placed on the animals' basic needs (e.g., food and/or water restriction, light cycles, temperature). Provide details regarding the length of restriction. Describe the method(s) for assessing the health and well-being of the animals during restriction. (Amount of food or fluid earned during testing and amount freely given must be recorded and assessed to assure proper nutrition.):

n/a

e. Describe how animals will be monitored (e.g., frequency of observations, by whom):

n/a

f. Analgesia (Category D Procedures) - list drugs, dosages, route of administration and frequency:

n/a

g. If treatment-related deaths are expected, this must be thoroughly justified. Death as an endpoint is highly discouraged:

n/a

7. Surgical Category D and E procedures. Indicate if the surgery is survival or terminal. Describe each surgical procedure separately, include details on the following (Also fill in Section B.9)

a. Complete description of surgical procedure including presurgical preparation, aseptic technique, surgical closure, etc:

none

b. Anesthetic regimen (Drugs, dosages, volume, route of administration and delivery schedule). The use of paralytic or neuromuscular blocking agents w/o anesthesia is prohibited:

n/a

c. Postoperative care (thermal support, special feeding, responsible personnel, removal of sutures/staples, frequency and duration of monitoring including weekend and holiday care):

n/a

d. Post operative analgesics (drugs, dosage, and volume and route of administration, frequency):

n/a

e. Will any animal be subject to more than one surgical procedure over the course of its lifetime, either here at NHEERL or elsewhere?

○ Yes ● No

f. Identify any surgical procedures performed at other institutions or by vendors:

none

8. Humane interventions (for treatments/procedures in all categories).

a. What resultant effects, if any, do the investigators expect to see following procedures or treatment? Please include transitory as well as permanent effects. Examples might include lethargy, ataxia, salivation or tremors. Indicate the expected duration of these effects.

Pre-eclampsia can be a life threatening situation if left untreated and allowed to develop into eclampsia (severe hypertension, convulsions, coma, and death). Both L-NAME and ozone has been successfully delivered throughout pregnancy at the doses proposed in this study without the progression to eclampsia and death. However, rats will be monitored daily throughout the experiment (including weekends and holidays) for overall health and temperament.

Additional potential deleterious effects to be monitored as related to disease outcomes and exposures:

- Reduced water consumption/ dehydration,
- Extreme changes in urine including amount/ frequency, foul smell, and blood and porphyrin discharge in urine,
- Residual urine staining around the perineum,
- Hyperirritability and depression; may be indicative of severe high blood pressure and stroke.In the event of expected or unexpected deleterious effects, the Attending Veterinarian will be immediately notified for guidance on subsequent steps including euthanasia. Animals will be isolated in a clean control atmosphere and observed for recovery trends, and may be transferred to the training colony if recovered. No deleterious effects of these non-surgical procedures, however, are expected as previous research has successfully published the proposed treatment protocols in pregnant rats without any reported deleterious effects.
- Porphyrin staining at eyes/nares,
- Starry coat (piloerection),
- Saliva staining on chin,
- Wet fur around muzzle,
- Behavioral abnormalities,
- Increased respiratory rate and effort.

b. State the criteria for determining temporary or permanent removal of animals from the study. Describe actions to be taken in the event of deleterious effects from procedures or chemical exposures. Describe actions to be taken in the event of clinical health problems not caused by procedures or exposures.

Any animals displaying signs of illness (weight loss of >10% occurs overnight, huddling, isolation with ruffled exterior, shivering, development of hindered movement, labored breathing and isolation, premature birth, vaginal bleeding, pale mucous membranes, dehydration, seizures and tremors, indications of severe high blood pressure and/or stroke, etc) will be considered for permanent removal as per advice of the staff veterinarian.

9. Alternatives to pain and distress (Category D and E Procedures only). Provide narrative regarding the sources consulted to ascertain whether acceptable alternatives exist for potentially painful/distressful procedures. Include databases searched or other sources consulted, the date of the search and years covered by the search, and key words and/or search strategy used. Assistance with searches is available through the EPA Library Staff.

n/a

SECTION C - Animal requirements

Describe the following animal requirements :

1. Indicate the number of animals required over the study period for this protocol. Please enter numbers only.

a. Animals to be purchased from a Vendor for this study: 90

b. Animals to be transferred from another LAPR:

LAPR Number that is the source of this transfer:

c. Animals to be transferred from another source:

d. Offspring produced onsite (used for data collection and/or weaned):

e. TOTAL NUMBER of animals for duration of the LAPR 90

2. Species (limited to one per LAPR): Rat(s)

3. Strain: Long Evans Time Pregnant Rat(s)

Describe special requirements for animals with altered physiological responses (e.g., genetically altered, aged)

Animals will be timed-pregnant rats that will arrive at gestational day 3 and staggered as needed to have manageable numbers for assays. This gestational age allows us to investigate the effect of ozone exposure during the majority of gestation and gives us enough confidence that the majority of rats (90%) are pregnant when they arrive at the facility.

Rats will be paired housed for the majority of their stay in the facility. Beginning on gestational day 16, pregnant rats will require single housing with the addition of nesting material in their home cage. While the pregnant rats will be euthanized prior to delivery (gestational day 21), rats will be permitted to build a nest as to not cause undue stress. As this study will be followed up with additional work, we request single housing at the later stage of pregnancy to mimic what will be done if rats were permitted to birth with pups easily identifiable to their dam. Cage changes will occur as usual, but may occur more frequently if needed.

Rats will be either maintained on the automatic water supply, in the case of Objective 2, or by providing glass water bottles in the case of Objective 1 (specifically the L-NAME experiment). All rats will be maintained on a purified diet that is modified for fat to meet the American Institute of Nutrition's guidelines for growth and gestation. The fat content in the basal diet (D12450H; Research Diets, Inc, New Brunswick, NJ) will be increased from 10% to 16% kcal from fat. The purpose for the use of the purified diet is to eliminate exogenous estrogens (phytoestrogens) that are known to impact both the cardiometabolic and reproductive systems. Further, this purified diet serves as the control diet for D12451 (Research Diets, Inc, New Brunswick, NJ), a 45% high fat diet that reliably induces obesity over time in Long-Evans rats. Future research with this model may include the use of a high fat diet during pregnancy or in the offspring and therefore it is important to use to the appropriate control diet at this phase as well. Signs will be hung on holding room doors to remind staff that a special diet is being provided to the rats.

4. Sources of animals:

Charles River Laboratories, Inc.

5. Provide room numbers where various procedures will be performed on animals:

1. Rats will be housed in one of the animal housing rooms upon arrival [redacted] or other available room) and during non-exposure periods.
2. During exposure, rats will be transferred in an original rack with rats housed in home cages to green floor inhalation exposure rooms (whole body exposures – [redacted]. Once the exposures are complete rats will be transferred to their home cages in the same rack and moved back to the animal holding room.
3. Urine will be collected in the animal housing room [redacted] or other available room).
4. Blood pressure will be obtained in room [redacted] or other available room.
5. Midpoint ultrasounds will be performed in room [redacted]
6. The day of ultrasound and necropsy, animals will be transferred to [redacted] for ultrasound and [redacted] (or other available room) for necropsy using transfer cages with beta chips bedding and filtered cage tops.

6. Will any animals be housed in areas other than the animal facility longer than 12 hours? If so, state location. Such areas require prior IACUC approval as a satellite facility before LAPR can be reviewed.

No *Room Numbers:*

7. Describe any transportation and containment methods involved in moving animals between EPA buildings, or between EPA and other institutions (excluding any commercial shipments)

none

8. Describe any unusual housing or husbandry requirements, or acclimation requirements. Justify any treatment beginning less than 3 days after arrival.

Because the current study seeks to investigate the effects of ozone exposure throughout pregnancy, rats must be quickly acclimated to the facility prior to initiation of the study. To do this, rats will be given a full day to acclimate, regardless of the time of day they are received at the facility. On their second full day in the facility, rats will be introduced to the restraining tube, however no measurement of blood pressure will be performed. Body weights and food intake will begin to be monitored daily upon their arrival and to the completion of the study.

9. Describe special assistance requested of the animal contract staff, including procedures and dosing. NOTE, this request must be submitted separately to the Animal Resources Program Office (ARPO)

We will request that the pregnant rats be provided with the appropriate nesting material in their home cage so they may be permitted to nest, particularly in the later stage of pregnancy. During Objective 1, rats enrolled within the L-NAME experiments will require glass water bottles.

10. Housing and Enrichment.

The IACUC encourages the use of environmental enrichment whenever possible (see IACUC website for details). Provide details on how the animals will be housed, including type of cage (e.g., solid bottom or wire screen), bedding material, number of animals per cage, and environmental enrichment. Note that housing rodents individually without environmental enrichment requires justification.

All animals will be pair housed in solid bottom caging with beta chips bedding in A building during non-exposure periods as per the IACUC guidelines. At gestational day 16, rats will be separated into single housing cages. The purpose of this is to mimic protocols for future research that involves allowing the rat to birth and keeping individual litters intact. Environmental enrichment including compressed bedding (Nestlets) and crinkled paper (EnviroDry) will be provided to allow the pregnant rats to nest.

SECTION D - Agents Administered to Animals

1. Identify all hazardous and non-hazardous agents to be administered to living animals. For agents requiring a Health and Safety Research Protocol (HSRP), provide the title of the approved HSRP for each such agent. If no protocol is required for an agent deemed potentially hazardous (e.g. nanoparticles, recombinant DNA), describe the safety precautions to be used.
Provide maximum dosing levels and route-appropriate LD50s (where available) for each agent used for dosing.

1) L-NAME to induce pre-eclampsia:

Toxicological information and LD50 was not found for L-NAME. We are, however, using doses found in the literature that successfully develops our model but does not result in death in rats (Podjarny et al; Hypertension. 1997). L-NAME will be delivered via the drinking water at a dose of 100 mg/L which has been found to be palatable by rats as observed in our previous studies using this compound Exemption 6 Exemption 6Exemption 6 . L-NAME is ordered as an ≥ 98% pure powder and must be stored as a powder for long term storage, thus, the final solution containing L-NAME will have to be made in house (every three days). L-NAME has no effect on the pH of water and thus administered drinking water will be within the range of standard tap water systems of pH 6 – 8.5.

2) Ozone inhalation exposures: Ozone exposure will occur in whole body exposure chambers to a maximum of 0.8 ppm concentration.

The LC50 for ozone is 4.8 ppm in rats (4800 ppb/ 4 hours/ inhalation/ rat).

3) Modified diet (D12450H; Research Diets, Inc.):

The diet used in this study is a purified diet free of phytoestrogens and is used as a control diet for a 45% high fat diet (D12451; Research Diets, Inc.). The stock diet contains 10% kcals from fat, which is lower than what is recommended for a pregnancy diet, thus the modified diet will be adjusted to provide 16% kcals from fat.

No LD50 exists for dietary total or saturated fat. Because the fat from this diet comes from lard, palmitic acid is the most concentrated lipid source. The oral LD50 for pure palmitic acid is > 10 g/kg.

HSRPs are not required for any of the agents listed.

No deleterious effects of these exposures are expected. Researchers will handle all agents in accordance with good industrial hygiene and safety practices. All animals will be monitored periodically during exposure.

4) Isoflurane: Exposure to isoflurane will occur in an induction chamber and through a nose cone contained within a fume hood at 1% - 3% isoflurane in oxygen delivered at 0.8 – 1.0 L/min.

The LD50 for inhaled isoflurane is 15300 ppm in rats.

5) Nair: A small amount of Nair will be added dermally to the ventral region of the rat for 1 -2 minutes and wiped off.

The LD50 for the active ingredient in Nair, potassium thioglycolate, has not been identified. Nair is a skin irritant and thus will only be used for the smallest time possible. Nair has been used successfully in our previous research without irritation to the rodent.

2. Describe compounds to be administered to animals.

a. Are all substances pharmaceutical grade? If not, provide a scientific justification for the use of non pharmaceutical grade compounds.

1) L-NAME to induce pre-eclampsia:

L-NAME is not available as a pharmaceutical grade chemical.

b. Describe any plans to administer human or animal tissues, blood or body fluids to the animals in the LAPR. Provide information to assure that such material is pathogen free. Indicate what safety precautions are necessary for handling the material.

No

c. Provide a statement regarding any safety precautions necessary for handling any of these materials.

n/a

NOTE: Any unresolved health/safety questions which arise during IACUC review of this LAPR will require consultation with the Safety, Health, and Environmental Management Office.

SECTION E - Personnel Training and Experience

1. Identify all project personnel conducting animal experimentation. Specify the techniques for which they have responsibility, and their relevant training and experience. Additional personnel may be added to the table below as a group (by Division) for Category C procedures. By so doing you are giving assurance that these personnel have received all required training and are qualified to perform the Category C techniques requested.

Use this area to type in additional personnel information not available in the table drop-down lists:

Hint: *The names in the first 2 lines of the table below are filled automatically from the Principal Investigator & Alternate Contact fields. A new line will be made available when a name is selected & upon leaving the name field (i.e. tabbing or clicking in another field).*

NAME	ROLE	SPECIFIC RESPONSIBILITY	RELEVANT TRAINING
Exemption 6	Principal Investigator	Plan study, prepare protocols, and oversee the experiment. Assist in animal handling, testing, and necropsy.	Thirty years of experience working with rats at EPA and other institutions, all required NHEERL training completed.
Exemption 6	Post-Doc	Plan study, prepare protocols, and oversee the experiment. Assist in animal handling, testing (tail blood pressure and ultrasound), and necropsy.	Eight years of experience working with rats at various institutions including pregnant Long-Evans rats. All required NHEERL animal training courses are completed.
Exemption 6	Associate Principal Investigator	Plan study, prepare protocols, and oversee the experiment. Assist in animal handling, testing, and necropsy.	30 years as a Veterinarian, and 37 years of experience in laboratory animal research. All required NHEERL animal training courses are completed.
Exemption 6	Associate Principal Investigator	Plan study, prepare protocols, and oversee the experiment. Assist in animal handling, testing, and necropsy.	More than 10 years of animal handling experience. All required NHEERL animal training courses are completed.
Exemption 6	Associate	Plan study, prepare	Seventeen years of experience working with

	Principal Investigator	protocols, and oversee the experiment. Assist in animal handling, testing, and necropsy.	rats and all required NHEERL animal training courses are completed.
Exemption 6	Post-Doc	Plan study, prepare protocols, assist in animal handling, ultrasound testing, and necropsy.	Six years of experience working with rats and has completed required animal training courses at NHEERL.
Exemption 6	Technical Staff	Assist in study planning, animal handling, and testing blood pressure.	Thirty years of experience working with rats and mice at NHEERL and other institutions, all required training completed.
Exemption 6 Exemption 6 Exemption 6	Technical Staff	Assist in study planning, animal handling, and necropsy.	Thirty years of experience working with rats and mice at NHEERL and other institutions, all required training completed.
Exemption 6	Technical Staff	Assist in study planning, animal handling, and necropsy.	Thirty years of experience working with rats and mice at NHEERL and other institutions, all required training completed.
Exemption 6	Technical Staff	Assist in study planning, animal handling, and necropsy.	Thirty years of experience working with rats and mice at NHEERL and other institutions, all required training completed.
RTP-NHEERL	Tech Support	Category C Procedures	All NHEERL required training is complete.

SECTION F - Animal Breeding Colonies

This section pertains to the breeding of animals for maintenance of ongoing animal colonies. Do not include breeding that is part of experimentation and accountable under Section C.

Describe:

1. Estimated number of breeding pairs and liveborn per year n/a

2. Breeding protocols and recordkeeping n/a

3. Methods for monitoring genetic stability n/a

4. Disposition of all offspring and retired breeders that are not used in accordance with the procedures described in this LAPR n/a

SECTION G - Euthanasia

1. When will the animals be euthanized relative to experimental procedures?

Pregnant rats will be necropsied for blood samples and tissue collection following terminal anesthesia on gestational day 21 immediately following ultrasound measurements. The uterus will opened to obtain fetal tissue and placental samples. Fetuses will be immediately decapitated once removed.

2. Describe the euthanasia techniques:

Method(s): Euthanasia plus exsanguination

Agent(s): Pentobarbital injectable preparations, diluted with sterile saline to achieve maximum of 200 mg/ml concentration

Dose (mg/kg): Maximum 150-250 mg pentobarbital/kg

Volume: Maximum 0.75 ml/kg

Route: Intraperitoneal

Source(s) of information used to select the above agents/methods:
Veterinary Staff
IACUC

3. Provide justification and references for any euthanasia agent or method that is not consistent with recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (e.g., cervical dislocation or decapitation without anesthesia; cervical dislocation in rodents weighing more than 200 grams).
None

4. Describe how death is to be confirmed.
Vital organ section

SECTION H - Disposition of Used and Unused Animals

Describe the disposition of any animals remaining after project completion.
Transferred to another study
The IACUC encourages investigators to reduce the overall number of animals used at NHEERL. Would you consider transferring any unused animals from this LAPR to another approved LAPR?
● Yes ○ No

SECTION I - Assurances

1. Animals will not be used in any manner beyond that described in this application without first obtaining formal approval of the IACUC.
2. All individuals involved in this project have access to this application, are aware of all EPA policies on animal care and use, and are appropriately trained and qualified to perform the techniques described.
3. Thorough consideration of the three "R"'s (Replacement, Reduction, Refinement) has been given, as applicable, to a. the use of animals, and b. procedures causing pain or distress (with or without analgesia/anesthesia), including death as an endpoint. The minimum number of animals required to obtain valid experimental results will be used.
4. The Attending Veterinarian has been consulted in regard to any planned experimentation involving pain or distress to animals.
5. The IACUC and Attending Veterinarian will be promptly notified of any unexpected study results that impact the animals' well-being, including morbidity, mortality and any occurrences of clinical symptoms which may cause pain or indicate distress.
6. All procedures involving hazardous agents will be conducted in accordance with practices approved by the Safety, Health, and Environmental Management Office.
7. I certify that I am familiar with and will comply with all pertinent institutional, state and federal rules and policies.
8. The IACUC has oversight responsibilities for animal care and use, and may request consultation or feedback regarding the conduct of in vivo procedures, progress and accomplishments, and any problems encountered.

EPA Principal Investigator	Certification Signature Date
Exemption 6 Exemption 6	08/24/2015

Submitted: 08/24/2015

Certification:
Certification by EPA Supervisor (Branch Chief or Division Director) that the project described herein has been reviewed and approved on the basis of scientific merit:

Branch Chief/Division Director	***Approval Date***	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	08/26/2015 by Exemption 6 /RTP/USEPA/US	Exemption 6 ***Lotus Notes Address*** Exemption 6 /RTP/USEPA/US	EPHD ***Branch*** CIB	MD ***Submitted to Branch Chief for Approval*** 08/24/2015 08:18 AM

ATTACHMENTS

Exemption 6 .pdf Study design.docx 18-08-003 LAPR PI Resp.pdf 18-08-003 PI Resp 2.pdf

Actions

First Update notification sent: 06/29/2016
Second Update notification sent: 07/27/2016
First 2nd Annual notification sent: 07/03/2017
Second 2nd Annual notification sent: 07/31/2017
1st Expiration notification sent: 06/28/2018
2nd Expiration notification sent:

History Log: